EXHIBIT B

UNIT 6.3

Using DNA Fragments as Probes

BASIC PROTOCOL

HYBRIDIZATION IN FORMAMIDE

Materials (*see APPENDIX 1* for items with ✓)

Nitrocellulose membrane filters bearing plaques, colonies, or DNA (*UNITS 6.1 & 6.2*)
✓ Hybridization solution I
Radiolabeled probe, 1 to 15 ng/ml (*UNIT 3.5*)
✓ 2 mg/ml sonicated herring sperm DNA
✓ Low-stringency wash buffer I
✓ High-stringency wash buffer I, prewarmed

1. Wet filters in turn with 5 to 20 ml hybridization solution I, producing a stack of 10 to 20 filters. Transfer to a sealable bag and add enough hybridization solution to cover. Seal and prehybridize ≥1 hr at 42°C.

   No more than ten 20 × 20–cm square filters or twenty 82-mm discs should be placed in one stack.

2. Boil radioactive probe (1 to 15 ng/ml hybridization reaction at >5 × 10^7 cpm/mg) with 2 mg (1 ml) sonicated herring sperm DNA for 10 min in a screw-cap tube.

3. Transfer to ice and add 2 ml hybridization solution I. Add probe mixture via syringe with 18-G needle to filters, reseal, mix thoroughly, and incubate overnight at 42°C.

4. Rinse filters three times with 500 ml low-stringency wash buffer I, room temperature, 10 to 15 min for each rinse.

   CAUTION: *The solution is extremely radioactive; handle carefully.*

5. Rinse filters twice with 500 ml high-stringency wash buffer I (prewarmed to wash temperature), 15 to 20 min for each rinse.

   Determine wash temperature empirically. If homology between probe and target approaches 100%, use a high-temperature wash of 65° to 75°C. For low homology and short probe lengths, lower temperature to 37° to 40°C. Wash very short probes <100 bp at lower temperatures regardless of homology.

6. Mount filters wet wrapped in plastic wrap or dry on plastic backing (e.g., used X-ray film) for autoradiography.

ALTERNATE PROTOCOL

HYBRIDIZATION IN AQUEOUS SOLUTION

Additional Materials (*see APPENDIX 1* for items with ✓)

✓ Hybridization solution II
✓ Low-stringency wash buffer II
✓ High-stringency wash buffer II

1. Prehybridize as in Basic Protocol except incubate in hybridization solution II at 65°C.

Prepare probe as in Basic Protocol and dilute with 2 ml hybridization solution II. Hybridize overnight at 65°C. Remove hybridization solution and rinse twice with low-stringency wash buffer II.

3. Wash filters quickly 5 to 8 times with high-stringency wash buffer II at 65°C. Leave in final wash ~20 min. Washed filters should produce a nonspecific signal only a few-fold above background levels.

References: Church and Gilbert, 1984; Denhardt, 1966.

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UNIT 6.4

Using Synthetic Oligonucleotides as Probes

BASIC PROTOCOL 1

HYBRIDIZATION IN SODIUM CHLORIDE/SODIUM CITRATE (SSC)

Materials (*see APPENDIX 1* for items with ✓)

Membrane filters bearing plasmid, bacteriophage, or cosmid libraries (*UNITS 6.1 & 6.2*)
3× SSC/0.1% SDS
✓ Prehybridization solution
✓ SSC hybridization solution
6× SSC/0.05% sodium pyrophosphate, prewarmed

1. Prepare duplicate nitrocellulose filters (processed and baked) of bacterial colonies or bacteriophage plaques. Wash 82-mm filters 3 to 5 times in 500 ml 3× SSC/0.1% SDS (50 filters) at room temperature. Then wash at 65°C at least 1.5 hr to overnight.

   Use filter forceps (without serrated tips) to handle the membrane filters.

2. Prehybridize in prehybridization solution 1 hr at 37°C.

3. Transfer up to 20 filters into sealable bags containing ≥20 ml SSC hybridization solution, and add 0.125 ng (for bacterial colonies) to 1.0 ng (for bacteriophage plaques) of each ^{32}P-labeled oligonucleotide/ml hybridization solution in one bag. Hybridize oligonucleotides 14 to 48 hr at the temperatures indicated:

   14-base—room temperature
   17-base—37°C
   20-base—42°C
   23-base—48°C.

4. Remove filters and wash three to five times in 6× SSC/0.05% pyrophosphate 5 to 15 min at room temperature. Wash 30 min in prewarmed 6× SSC/0.05% pyrophosphate at the temperatures indicated:

   14-base—37°C
   17-base—48°C
   20-base—55°C
   23-base—60°C.